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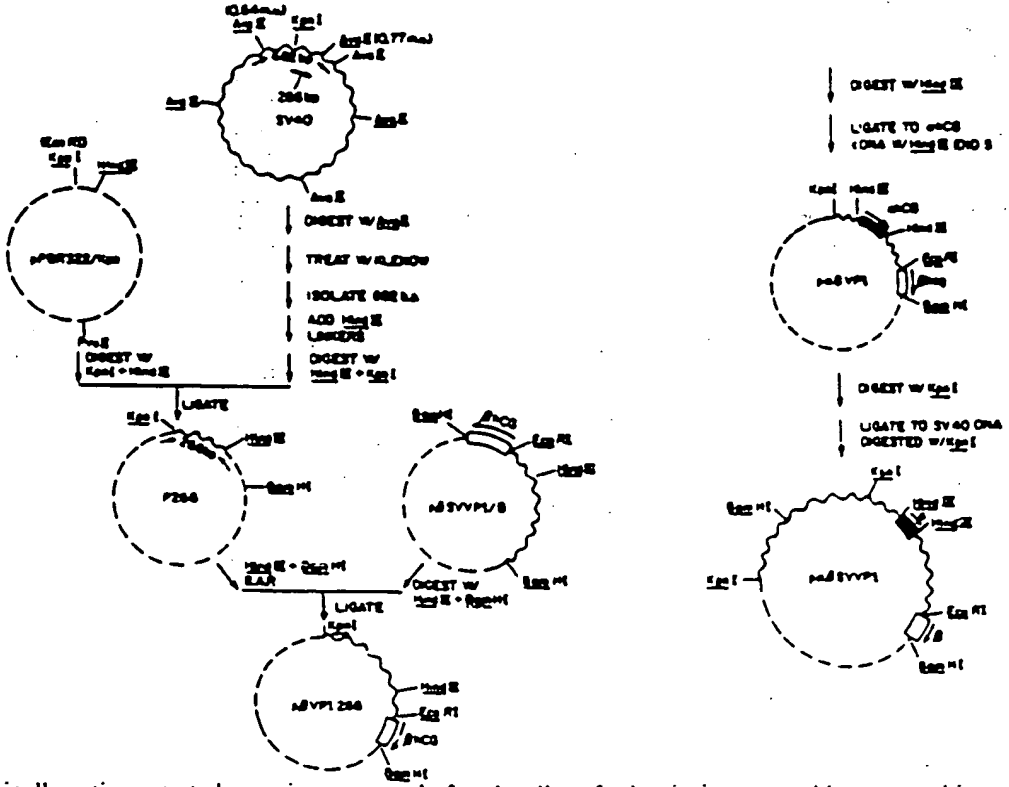
(54) Title: PRODUCTION OF HETERODIMERIC HUMAN FERTLITY HORMONES



(57) Abstract

A biologically active secreted protein composed of a plurality of subunits is prepared by a recombinant methodology. Each subunit is synthesized by a cell having an autonomously replicating expression vector I.E. a plasmid or virus containing heterologous DNA encoding for the subunit. The preferred host is a eukaryotic cell. The secreted protein is a hormone such as hCG, luteinizing hormon (LH), follicle stimulating hormone (FSH) or thyroid stimulating hormone (TSH).

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PRODUCTION OF HETERODIMERIC HUMAN FERTILITY HORMONES

Background of the Invention

This invention relates to the use of recombinant DNA techniques to produce heteropolymeric proteins.

Various polypeptide chains have been expressed, via recombinant DNA technology, in host cells such as bacteria, yeast, and cultured mammalian cells. Fiddes, J. C. and Goodman, H. M. (1979) Nature Vol. 281, pg. 351-356 and Fiddes, J. C. and Goodman, H. M. (1980) Nature Vol. 286, pg. 684-687 describe the cloning of, respectively, the alpha and beta subunits of human choriogonadotropin (hCG).

Kaname US Pat. 4,383,036 describes a process for producing hCG in which human lymphoblastoid cells are implanted into a laboratory animal, harvested from the animal, and cultured in vitro; accumulated hCG is then harvested from the culture.

Summary of the Invention

In general, the invention features, in one aspect, a biologically active heteropolymeric protein composed of a plurality of subunits, each subunit being synthesized by a cell having an autonomously replicating (i.e., not integrated into the chromosone of the host cell) expression vector containing heterologous DNA encoding the subunit.

In preferred embodiments, the protein is synthesized by a eukaryotic cell and the protein is modified post-translationally, most preferably by glycosylation; and the protein is a secreted protein such as a hormone, most preferably a fertility hormone such as hCG, luteinizing hormone (LH) or follicle stimulating hormone (FSH); or the hormone thyroid stimulating hormone (TSH).

In another aspect, the invention features a cell, containing a first expression vector, which cell is capable of producing a biologically active heteropolymeric protein that is encoded at least in part by the vector. In preferred embodiments, a second autonomously replicating expression vector encodes a second portion of the protein or at least two subunits of the protein are encoded by a single expression vector; the protein is hCG or human luteinizing hormone (LH); the vector is a replicating virus or a plasmid; the cell is a monkey or mouse cell; transcription of the different hCG or LH subunits is under the control of the SV40 late promoter; transcription of the alpha subunit of the protein is under the control of the SV40 early promoter and transcription of the beta subunit is under control of the mouse metallothionein promoter, or transcription of both subunits is under the control of the mouse metallothionein promoter; and the expression vector which includes the mouse metallothionein promoter also includes at least the 69% transforming region of the bovine papilloma virus (BPV) genome.

In another aspect, the invention features an autonomously replicating expression vector including two genes encoding two different heterologous proteins, the genes being under the control of two different promoters, most preferably a metallothionein promoter and a BPV promoter; the use of different promoters advantagecusly minimizes the possiblity of deleterious recombinations.

As used herein, "subunit" refers to a portion of a protein, which portion, or homologue or analogue thereof, is encoded in nature by a distinct in mRNA.

Thus, for example, a heavy chain and a light chain of an IgG immunoglobulin are each considered a subunit. Insulin, on the other hand, is composed of two chains which are not considered subunits, because both are, in nature, encoded by a single mRNA, and cleavage into two chains naturally occurs only after translation.

The term "expression vector" refers to a cloning vector which includes heterologous (to the vector) DNA under the control of control sequences which permit expression in a host cell. Such vectors include replicating viruses, plasmids, and phages.

The invention permits the production of a biologically active heteropolymeric protein from a single culture of transformed cells. The production of both subunits of a heteropolymeric protein in the same cell eliminates the necessity of recombining subunits from separate cultures to assemble an active heteropolymeric molecule. The system also allows production of proteins, in a single culture, which undergo, in the culture, post-translational modification, e.g. glycosylation and proteolytic processing, for activity or stability.

The use of autonomously replicating expression vectors prevents undesirable influence of the desired coding regions by control sequences in the host chromosone.

Other advantages and features of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

We turn now to the preferred embodiments of the invention, first briefly describing the drawings thereof.

Drawings

Fig. 1 is a diagrammatic illustration of the construction of the plasmid p alpha SVHVP1, which contains the alpha hCG cDNA clone, portions of SV40 viral DNA, and sequences of the plasmid pBR 322.

Fig. 2 is a diagrammatic illustration of the construction of plasmid p beta SVVP1, which incorporates the beta hCG cDNA clone, regions of SV40 DNA and a portion of pBR 322 including the region conferring resistance to ampicillin on host E. coli.

Fig. 3 is a diagrammtic illustration of the construction of the plasmid p alpha beta SVVP1 in which the alpha and beta hCG cDNA clones are inserted into SV40 DNA.

Fig. 4 is a diagrammtic illustration of the construction of the plasmids pRF375 and pRF398.

Fig. 5 is a diagrammatic illustration of the construction of the plasmid RF398 alpha t_2.

Fig. 6 is a diagram illustrating the location of an 88 bp probe within the beta hCG cDNA clone.

Fig. 7 illustrates the beta LH restriction map, and the pieces used in the construction shown in Fig. 8.

Fig. 8 is a diagrammatic illustration of the construction of a plasmid, LH520H/B, containing the complete mature beta LH cDNA clone.

Fig. 9 is a diagrammatic illustration of the construction of the viral vector p alpha LHSVVP1.

Fig. 10 is a diagrammatic illustration of the construction of the BPV-containing plasmid pCL28XhoLHBPV, encoding the beta subunit of LH.

Structure

The cloning vectors of the invention have the general structure recited in the Summary of the

Invention, above. Preferred vectors have the structures shown in the Figures, and are described in more detail below.

Construction of Cloning Vectors

Isolation of cDNA Clones Encoding the Alpha and Beta Subunits of hCG

All of the techniques used herein are described in detail in Maniatis et al. (1982) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory), hereby incorporated by reference.

RNA is extracted from placental tissue by the following method. Homogenization of the tissue is carried out in a 1:1 mixture of phenol:100mM Na-acetate (pH 5.5) containing 1mM EDTA, that has been warmed to 60° for 20 min. After cooling on ice for 10 min., the phases are separated by centrifugation. The hot phenol extraction is repeated twice more followed by two extractions with chloroform.

RNA is precipitated from the final aqueous phase by the addition of 2.5 volumes of ethanol.

In order to enrich for poly A+ mRNA, placental RNA is passed over oligo (dT)-cellulose in 0.5M NaCl buffered with 10mM Tris-Hcl, pH 7.5, and washed with the same solution. Poly A+ mRNA is eluted with 10mM Tris-HCl (pH 7.5), 1mM EDTA, 0.05% SDS and precipitated twice with ethanol. Typical initial yields are 1.5-2.0 mg of total RNA per g of tissue, of which about 2% is poly A+ mRNA.

Placental cDNA libraries are constructed by reverse transcription of placental mRNA, second strand synthesis using E. coli DNA polymerase I (large fragment), treatment with SI nuclease, and homopolymer

tailing (dC) with terminal deoxynucleotidyl transferase; all such procedures are by conventional techniques.

In a typical preparation, 20-30% conversion of mRNA to single strand (ss) cDNA; 70% resistance to digestion with nuclease S1 after second strand synthesis; and dC "tails" of ten to twenty-five bases in length, are obtained. These cDNA molecules are then annealed to DNA fragments of the plasmid pBR 322, which has been digested with PstI, and to which dG "tails" have been added. These recombinant plasmids are then used to transform E. coli cells to generate a cDNA library (transformed cells are selected on the basis of tetracycline resistance).

In order to identify the human alpha hCG clone, a 219 bp fragment of a mouse alpha thyroid stimulating hormone (TSH) clone is used as a hybridization probe. This probe has 77% sequence homology with the human clone. It is radioactively labeled by nick translation and hybridized to the cDNA library under conditions that take into account the extent of homology. Strongly hybridizing clones are analyzed by restriction mapping and clones containing the complete coding sequence of alpha hCG are verified by DNA sequencing.

Construction of Plasmid p alpha SVHVP1

Referring to Fig. 1, in order to construct the plasmid alpha 970 H/B, a cDNA clone containing the alpha hCG fragment is digested with NcoI. The NcoI site, just 5' to the ATG codon signalling initiation of translation, is filled in and ligated to a synthetic HindIII linker. Similarly, the natural HindIII site in the 3' untranslated region of the clone is cut, filled in with E. coli DNA polymerase Klenow, and then ligated

to a synthetic BamHI linker. This fragment is cloned into the plasmid pBR 322 between its HindIII and BamHI sites to generate the plasmid alpha 574 H/B. This plasmid is digested with BamHI, treated with alkaline phosphatase, and ligated to the 396 bp Sau3A fragment of SV40 DNA (from 0.07 to 0.14 map units) which has been isolated from a polyacrylamide gel. The ligation mix is used to transform E. coli to ampicillin resistance and the desired plasmid, alpha 970 H/B, is identified among the transformants.

The plasmid $Q_2 7$ is constructed by cutting SV40 at its HpaII site, making flush ends by digestion with nuclease S1, ligating on Eco RI linkers, digesting with EcoRI, and cloning the resulting 1436 bp fragment into the EcoRI site of pBR 322.

Referring to Fig. 1, $Q_2 7$ is digested completely with EcoRI and partially with HindIII; the fragment from 0.72 to 0.94 map units is isolated and cloned into alpha 970 H/B, which has been digested with EcoRI and HindIII and treated with alkaline phosphatase. The ligation mix is used to transform E. coli, and the desired plasmid, p alpha SVL, is identified among the transformants by restriction mapping.

p alpha SVL is digested with EcoRI and the fragment of SV40, with EcoRI ends, extending from 0 to 0.72 map units, and containing the SV40 origin of replication and the intact early region, is ligated to it to generate the plasmid p alpha SVHVP1, which is isolated from E. coli transformants.

Construction of Plasmid p beta SVVP1

A 579 bp cDNA clone coding for beta hCG was obtained from John C. Fiddes at Cold Spring Harbor

Laboratory, Cold Spring Harbor, New York (Fiddes et al (1980) Nature Vol. 286, pg. 684-687). T is fragment is ligated at each end to synthetic BamHI linkers. After digestion by HgaI restriction enzyme, the ends are filled in with Klenow DNA polymerase and synthetic EcoRI linkers are ligated on so that an EcoRI site is about 10 bp 5' to the ATG codon of the signal peptide coding sequence. A BamHI site is about 60 bp 3' to the nonsense codon marking the end of the coding sequence. Referring to Fig. 2, this 556 bp EcoRI-BamHI fragment is isolated and cloned into pBR 322, between the EcoRI and BamHI sites, to give the plasmid p beta 556 R/B.

In order to construct the plasmid pSVHR (Fig. 2), SV40 DNA is partially digested with HindIII to yield linear molecules, digested with nuclease S1 to make flush ends, ligated to synthetic EcoRI linkers and digested with EcoRI and BamHI. The fragment from 0.94 to 0.14 map units, containing the SV40 origin of replication and early region, is cloned into pBR 322 as an EcoRI-BamHI piece.

Referring still to Fig. 2, the EcoRI site of the plasmid p beta 556 R/B is methylated in a reaction catalyzed by EcoRI methylase, following which the plasmid is cut with NdeI. EcoRI linkers are ligated to the SI treated NdeI flush ends and activated by digestion with EcoRI, which is followed by digestion with BamHI.

The SV40 fragment of pSVHR from the EcoRI site to the BamHI site is isolated and ligated in a reaction mix containing the digestion fragments of p beta 556 R/B. Following ligation, the mix is digested with SalI to eliminate plasmids which have re-inserted the EcoRI

(NdeI) to BamHI piece of pBR 322. E. coli is transformed with the digested ligation mix and p beta SVVP1 is identified and isolated.

Construction of the Plasmid p Alpha Beta SVVP1

Referring to Fig. 3, pBR 322/Kpn is derived from pBR 322 by inserting a KpnI linker into its unique EcoRI site, after this site is deleted by digestion with EcoRI, followed by digestion with S1 nuclease.

Referring still to Fig. 3, SV40 DNA is digested with AvaII. The staggered ends of the resulting fragments are filled in by Klenow DNA polymerase to form flush ends, and the mixture is then fractionated on a polyacrylamide gel. The 682 base pair fragment (0.64 to 0.77 map units) containing the origin of replication and the unique KpnI site is isolated from the gel, ligated to synthetic HindIII linkers, and digested with HindIII and KpnI.

The resulting fragments are ligated to pBR 322/Kpn. p266, which contains the 266 base pair KpnI - HindIII fragment, including the SV40 late promoter region, is isolated. p266 is cut with HindIII and BamHI, and treated with bacterial alkaline phosphatase.

Still referring to Fig. 3, p beta SVVPI/B is constructed as follows: p beta SVVPI (Fig. 2) is cut with EcoRI, followed by ligation to eliminate pBR 322 sequences. Subsequently, this DNA is cut with BamHI and cloned into the BamHI site of pBR 322.

The resulting plasmid, p beta SVVPI/B, is then digested with HindIII and BamHI and the 1003 base pair HindIII-BamHI fragment is ligated into p266 to yield the plasmid p beta VP1 266, in which the beta hCG cDNA is positioned downstream from the SV40 late promoter in

such a way that its RNA transcript would be spliced as if it were the viral VP1 transcript.

The alpha hCG cDNA is inserted into p beta VP1 266 as a HindIII fragment, which has been cut at its HindIII site and treated with bacterial alkaline phosphatase. E. coli transformants derived from this ligation are screened by restriction mapping, and plasmids are isolated that have the desired structure, in which the alpha hCG cDNA has replaced VP2 in the correct orientation, followed downstream by the beta hCG cDNA, which has replaced VP1.

One such isolated plasmid, p alpha beta VP1, is used to complete the construction of p alpha beta SVVP1. The plasmid is cut with KpnI, and the full SV40 genome, cut with KpnI, is inserted by ligation into this site. Following transformation of E. coli, a plasmid with the required structure, p alpha beta SVVP1, is isolated. This plasmid contains DNA encoding both the alpha and beta subunits of hCG, and thus is capable of directing the expression, in host mammalian cells, of both subunits, whereby biologically functional, glycosylated heterodimeric hCG is produced (glycosylation occurs post-translationally).

Construction of Plasmids pRF 375 and pRF 398

Referring to Fig. 4, the plasmid CL28 (identical to plasmid JYMMT(E); Hamer et al. (1983) J. Mol. Applied Gen. 1, 273-288), containing the murine metallothionein promoter, SV40 DNA, and pBR 322 sequences, is cut with the restriction endonuclease Bgl II. At this site are inserted cDNA clones of either alpha hCG or beta hCG, containing untranslated regions of about 10 and 30 bp at their 5' and of about 220 and

60 bp at their 3' ends. These clones have been genetically engineered by the addition of synthetic Bam HI linkers at their termini.

The resulting plasmids pRF 302 (alpha) or pRF 394 (beta) are digested with restriction enzymes BamHI and SalI to release the SV40 DNA sequences.

Plasmid pB2-2, which contains the entire BPV genome, and some pBR 322 sequences, is digested with BamHI and SalI to yield the BPV genome with BamHI/SalI ends; this fragement is ligated into pRF 302 (alpha) and pRF 394 (beta) containing the metallothionein-hCG sequences.

Following transformation of E. coli, plasmids pRF 375 and pRF 398 are identified and isolated. They encode alpha hCG or beta hCG, respectively, under the control of the mouse metallothionein promoter.

Construction of the Plasmid RF 398 alpha t_2

Referring to Fig. 5, the plasmid p alpha t2 is derived by cloning the alpha hCG 574 HindIII fragment into plasmid pVBt2 (V.B. Reddy et al., PNAS 79, 2064-2067, 1982). p alpha t2, which contains the alpha hCG cDNA under the control of the SV40 early promoter, is digested with EcoRI. The 5' overhangs are removed by S1 nuclease digestion prior to the addition of synthetic BamHI linkers by blunt end ligation.

Plasmid RF 398 (Fig. 4) is digested with BamHI and treated with bacterial alkaline phosphatase. The 1735 base pair BamHI fragment of p alpha t_2 is inserted in to RF 398. The resulting plasmid RF 398 alpha t_2 is isolated from E. coli transformants. This plasmid thus has the beta hCG cDNA in a transcriptional unit under control of the mouse metallothionein promoter

and the alpha hCG cDNA in a transcriptional unit controlled by the SV40 early promoter.

Expression of Luteinizing Hormone (LH) cDNA Clones

Construction of a Human Pituitary cDNA Library

RNA is prepared from human pituitaries by homogenizing 5 to 10 grams of the frozen glands in 20 ml of a solution containing 4 M guanidine thiocyanate, 1 M 2-mercaptoethanol, 0.05 M Na-acetate (pH 5.0), and 0.001 M EDTA. One g CsCl is added per ml of homogenate and the suspension is centrifuged at 2,000 rpm for 15 min. The supernatant is layered carefully over a 15 ml cushion of CsCl solution (containing 1.25 ml of 1 M Na-acetate (pH 5), 62.5 microliters of 0.4 M EDTA and 39.8g of CsCl in a final volume of 35 ml) and centrifuged at 45,000 rpm in the Ti 70 rotor of a Beckman ultracentrifuge for 18-24 h at 20°C. The RNA visible as a pellicle in the gradient is removed with a syringe, diluted, and precipitated by the addition of two volumes of ethanol. Following three cycles of dissolution and reprecipitation, the RNA pellet is dissolved in H_2O and brought to 0.01 M Tris-HCl (pH 7.5) and 0.5 M NaCl by the addition of concentrated stock solutions. The preparation is then enriched for poly A^+ mRNA by two passes over oligo dT-cellulose, as described above in the case of placental RNA.

A human pituitary cDNA library is constructed from the poly A^+ mRNA as described above for placental poly A^+ mRNA except that both the large fragment E. coli DNA polymerase I and the avian myeloblastosis virus reverse transciptase are used sequentially for second strand cDNA synthesis. The Klenow is used first. The reaction is stopped by phenol extraction.

The aqueous phase of the centrifuged extract is applied to a 5 ml column of BioGel A-5m. Fractions containing high molecular weight material are pooled, concentrated, precipitated with two volumes of ethanol, dried, and dissolved in 100 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 140 mM KCl, 20 mM 2-mercaptoethanol, 1 mM of each of the four deoxyribonucleoside triphosphates, for reverse transcription. Reverse transcriptase is added to about 20 units per microgram of cDNA. Double stranded cDNA is then treated with nuclease S1, tailed, and cloned as described above.

Isolation of Beta LH cDNA Clones

Colonies grown on nutrient agar plates containing 25 micrograms per ml of tetracycline are transferred to nitrocellulose filters. Colonies are lysed *in situ* by treatment with 0.5 M NaOH and neutralized with 0.5 M Tris-HCl (pH 7.4) containing 1.5 M NaCl. Liberated DNA is fixed to the filter by baking at 80°C in a vacuum oven for 2 h. The filters are screened by hybridization to a ^{32}P labeled 88 base pair fragment of the beta hCG clone corresponding to amino acids 16 to 45 of the mature hCG beta chain, which has 29 of 30 amino acids in common with this region of the beta LH polypeptide (Fig. 6). Hybridization is carried out overnight at 32° in 50% formamide, 0.75 M NaCl, 0.075M Na-Citrate (pH 7.0), 2.5% dextran sulfate, 0.1% polyvinylpyrollidone, 0.1 mg per ml bovine serum albumin, and at least 10^5 cpm per filter of ^{32}P-labeled 88 bp beta hCG fragment. Filters are washed several times in 0.15 M NaCl, 0.015 M Na-citrate at 37°C before autoradiography. One of the positive isolated clones LH12 (Figure 7), is used further. LH12

is 365bp long and includes sequences coding for 15 amino acids of the pre-beta signal sequence plus 105 amino acids of the mature beta LH polypeptide. Its nucleotide sequence is determined. Since the complete mature beta LH is not coded by LH12, further screening of the human pituitary cDNA library is carried out using a 240 bp NcoI-PvuII fragment of LH12 (Fig. 7) as a ^{32}P labeled hybridization probe. The clone LH6 (Fig. 7) is isolated from this screening. LH6 contains the complete 3' end of beta LH, including the region corresponding to the untranslated portion of the mRNA through 27 A residues of the poly A "tail" of the mRNA. No clones are found that extended further than LH12 in the 5' direction. DNA sequencing of the complete, combined mature beta LH coding regions reveals two differences in the amino acid sequence of beta LH from the published protein sequence data: position 42 is a methionine and position 55 is a valine. Also, the mature beta LH contains 121 amino acids, based on the cDNA sequence.

A clone containing an intact signal peptide coding sequence and the complete mature beta LH sequence is constructed as shown in Fig. 8, using the restriction fragment illustrated in Fig. 7. A 104 bp EcoRI-DdeI fragment is isolated from the plasmid beta 579 H and ligated to an isolated 181 bp DdeI fragment, subsequently digested with PstI, from the LH12 plasmid. Following ligation overnight at 15°C, the ligation mix is digested with EcoRI and PstI and fractionated on a 7% polyacrylamide gel from which the desired 256 bp fragment is isolated. This fragment fuses the beta hCG signal sequence to that of the pre-beta LH in such a way as to provide a coding sequence for a 20 amino acid signal peptide.

The 256 bp EcoRI-PstI fragment is cloned into pBR 322 digested with EcoRI and PstI so as to give the plasmid LH beta 260. The 146 bp EcoRI-NcoI fragment indicated in Fig. 8 is isolated from a polyacrylamide gel and used later in the construction as described below.

The LH6 plasmid (Fig. 8) is digested with Sau3a and the 390 bp fragment is isolated by polyacrylamide gel electrophoresis. This fragment is then digested with HincII, ligated to BamHI linkers, digested with BamHI, and cloned into the plasmid pAPP at the BamHI site. pAPP is derived from pBR 322 by digestion with AvaI, filling in the 5' overhang with the dNTP's and the large fragment DNA polymerase I of E. coli, digestion with PvuII, and ligation to close the plasmid so as to eliminate the PvuII site. The plasmid LH6B, isolated from the ligation of the 340 bp BamHI fragment into the BamHI site of pAPP, is digested with EcoRI and PvuII, and treated with bacterial alkaline phosphatase. The fragments are ligated to a mixture of the 145 bp EcoRI-NcoI fragment of LH beta 260, described above, and the isolated 241 bp NcoI-PvuII fragment from the plasmid LH12 shown in Fig. 8. The ligation mix is used to transform E. coli to ampicillin resistance. The plasmid LH 520 H/B is found among the transformants. LH 520 H/B contains a complete beta LH coding sequence including a hybrid signal peptide sequence.

Construction of p Alpha LHSVVP1

In order to express this pre-beta LH clone in an SV40-based vector, as had been done for the pre-alpha and pre-beta hCG clones described previously, it is desirable to place an EcoRI site very close to the ATG

of the pre-beta coding sequence. This is accomplished by digesting LH520 H/B with HgaI, filling in the 5' overhang, ligating on synthetic EcoRI linkers, digesting with EcoRI and BamHI, and cloning the isolated 496 bp EcoRI-BamHI fragment into pBR 322 digested with EcoRI and BamHI and treated with bacterial alkaline phosphatase. The plasmid pLH496 R/B is isolated from E. coli transformed with this ligation mix and is used as the source of the 496 bp fragment to be expressed.

The plasmid p alpha beta VP1, whose construction and use in expressing both subunits of hCG is described earlier (Fig. 3), is digested with EcoRI and BamHI and ligated in a reaction mix containing the plasmid pLH496 R/B which had been digested with both of these enzymes (Fig. 9). The plasmid p alpha LHVP1 is identified among the E. coli transformants. As shown in Fig. 9, the intact SV40 viral early region is taken from p alpha SVHVP1 (Fig. 1) and inserted by ligation as a KpnI-SalI fragment into p alpha LHVP1 which had been digested with KpnI and SalI to give the plasmid p alpha LHSVVP1. By cutting this plasmid with BamHI and religating, the virus alpha LHSVVP1 is formed. This virus contains cloned cDNA's for the common (to LH and hCG, as well as FSH and TSH) alpha subunit and the specific beta LH subunit under control of the SV40 late promoter. The cloned cDNA's are positioned in such a way that the common alpha insert replaced the viral VP1 protein coding sequence and the beta LH insert replaced the viral VP2 coding sequence.

Insertion of the Beta LH cDNA (With Beta hCG 5' End of Signal Peptide) into a BPV-Based Expression System

LH 520 H/B (Fig. 8) is digested with HindIII and BamHI, treated with the E. coli DNA polymerase

(Klenow), ligated to synthetic SalI linkers, digested with SalI, and cloned into the SalI site of pBR 322. The resulting plasmid, LH 530 Sal, is used as a source of the LH cDNA clone for insertion into the mouse metallothionein gene of the plasmid CL28 as described in Fig. 10.

CL28 is cut with BglII, treated with nuclease S1, and ligated to XhoI linkers. Following digestion with XhoI, ligation and digestion with BglII, E. coli is transformed with the reaction mix to give the plasmid CL28Xho. This plasmid is digested with BamHI and SalI and ligated to a BamHI plus SalI digest of the plasmid pB2-2 (Fig. 4) to give the plasmid CL28XhoBPV. The latter LH insert is then ligated into the XhoI site of CL28XhoBPV as a SalI fragment, since the 5' overhang of SalI digests is complementary to that of XhoI digests. Following digestion with XhoI to eliminate background, E. coli is transformed and the desired plasmid pCL28XhoLHBVP containing the (hybrid) pre-beta LH insert, in a BPV-based plasmid, under control of the mouse metallothionein promoter, is isolated.

Transfection and Infection of Host Monkey Cells

The incorporation of virus-containing vectors into eukaryotic cells for the production of a heteropolymeric protein is generally accomplished as follows. First, if the viral DNA and homopolymeric protein-encoding DNA are incorporated into a plasmid, which is maintained, in, say, E. coli, the plasmid sequences (e.g. the pBR 322 sequences) are removed and the resulting DNA is ligated to form circular DNA including the viral region and the heteropolymeric protein-encoding sequence or sequences. This circular

DNA generally does not contain all of the genetic information needed to produce a replicating virus, the other necessary sequences (e.g. those encoding coat protein) having been replaced by the heteropolymeric protein-encoding sequence or sequences. The circular DNA, minus the plasmid DNA, must be close enough in size to the naturally occurring viral DNA from which it is derived to permit the DNA to enter and replicate in appropriate host mammalian cells.

The circular DNA is used to transfect host cells in order to produce virus stock for later infections. Since some of the DNA necessary to produce virus is missing, the transfection must occur in conjunction with helper virus DNA encoding enough of the missing function to produce replicating virus.

Transfected host cells are grown and incubated until lysed by replicating virus. The resulting replicating virus stock, including helper virus, is then used to infect host cells for production of the heteropolymeric protein. Virus stock is maintained, since it generally needs to be reused to infect fresh batches of host cells, as each culture of infected, protein-producing host cells generally is eventually lysed by the virus.

The specific recombinant DNA sequences described above are used to transfect, and then infect, host cells, as follows.

The pBR 322 sequences are removed from the above-described SV40-containing plasmids to produce transfecting viral DNA. In the case of p alpha SVHVP1 and p alpha beta SVVP1, this is accomplished by digestion with BamHI, followed by ligation under

conditions favoring circularization of the fragments to give (among other products) alpha SVHVP1 and alpha beta SVVP1. For p beta SVVP1, digestion with EcoRI followed by re-ligation brings the SV40 late promoter and VP1 splice region into juxtaposition with the beta hCG cDNA insert at the same time that it eliminates pBR 322 sequences and forms beta SVP1. At the same time, ptsA58 Bam (tsA58 SV40 viral DNA cloned into the pBR 322 BamHI site) is cut with BamHI and ligated to obtain self-ligated circles. Analogous methods are used for the LH vectors. Separate virus stocks are prepared as described below.

The DNA's, which are cut and ligated as described above, are ethanol precipitated and dissolved in sterile water. Approximately 1 ug of ptsA58 Bam DNA (helper virus) and 10 ug of recombinant DNA (encoding alpha and/or beta hCG or LH) are combined in a sterile test tube, mixed with 2 ml of TBS buffer (G. Kimura and R. Dulbecco 1972, Virogy, 49, 79-81) and 1 ml of 2 mg/ml DEAE-dextran solution and added to a monolayer of confluent monkey CV-1 cells previously washed twice with 10 ml of TBS in a T-75 flask. The cells are left at 37°C for 1-2 hrs with occasional shaking, washed with TBS twice, fed with 10 ml of DMEM containing 5% fetal calf serum, and left at 40°C for 10-15 days. After complete cell lysis, the medium is transferred to a test tube, frozen and thawed five times, and centrifuged at 3000 rpm for five minutes. The resulting supernatants serve as virus stocks for infection of fresh CV-1 cells.

To accomplish an infection, CV-1 cells are grown to confluence in a T-150 flask. 1 ml of one of the virus stocks (made as described above) is added to

the flask and the cells are incubated at 40°C for 5 days.

For mixed infections, CV-1 cells are grown to confluence in a T-150 flask. alpha SVHVP1 and beta SVP1 viruses are mixed in a 1:1 ratio and 1 ml of the mixed virus is used to infect CV-1 cells at 40°C.

Transfection of Mouse Cells

To produce heterodimeric hCG using a mixed transfection, five ug of each BPV plasmid, i.e., pRF 375 (alpha hCG) and pRF 398 (beta hCG), are mixed and added to 0.5 ml of a 250 mM $CaCl_2$ solution containing 10 ug of salmon sperm DNA as carrier. This mixture is bubbled into 0.5 ml of 280 mM NaCl, 50 mM Hepes and 1.5 mM sodium phosphate. The calcium phosphate precipitate is allowed to form for 30-40 minutes at room temperature.

24 hours prior to transfection, $5x10^5$ cells of mouse C127 cells (available from Dr. Dean Hamer, National Cancer Institute, NIH, Bethesda, MD) are placed in a 100 mm dish or T-75 flask. Immediately before adding the exogenous DNA, the cells are fed with fresh medium (Dulbecco's Modified Medium, 10% fetal calf serum). One ml of calcium phosphate precipitate is added to each dish (10 ml), and the cells are incubated for 6-8 hours at 37°C.

The medium is aspirated and replaced with 5 ml of 20% glycerol in phosphate buffered saline, pH 7.0 (PBS) for 2 minutes at room temperature. The cells are washed with PBS, fed with 10ml of medium, and incubated at 37°C. After 20-24 hours, the medium is changed and subsequent refeeding of the cells is carried out every 3-4 days. Individual clones are grown in T-25cm flasks. After 7-21 days, cell clones can be transferred to larger flasks for analysis.

To produce heterodimeric hCG using a single transfection, plasmid RF 398 alpha t_2 is employed in the same manner as the above two plasmids were employed for a mixed infection.

To make heterodimeric LH, plasmids PRF 375 and pCL28XhoLHBPV are mixed, as described above in the case of hCG.

An interesting observation is that culturing cells containing beta hCG or beta LH-encoding vectors alone, in the absence of alpha-encoding cells, produces practically no beta subunit, while cells containing alpha and beta-encoding sequences produce not only heterodimer, but free beta subunit as well. This lends support to the notion that the production of both subunits in a single cell culture has the additional advantage of somehow permitting the presence of the alpha subunit to stabilize the beta subunit.

Deposits

The following, described above, have been deposited in the American Type Culture Collection, Rockville, MD:

alpha beta SVVP1, ATTC VR2077 ;
alpha SVHVP1, ATCC VR2075 ;
beta SVVP1, ATCC VR2075 ;
pRF 375 in C127 cells, ATCC CRL8401 ;
pRF 398 in C127 cells, ATCC CRL8401 ;
pCL28XhoLHBPV *E. coli*, ATCC 39475 ;
pRF 398 alpha t_2 in C127 cells.

Use

The transformed cell lines of the invention are used to produce biologically active heteropolymeric proteins. hCG made according to the invention, for

example, has a number of well-known medical uses associated with human fertility.

Other Embodiments

Other embodiments are within the following claims. For example:

Other heteropolymeric proteins, e.g., follicle stimulating hormone, or thyroid stimulating hormone, can be produced, as can human or animal immunoglobulins or immune response antigens.

It may not be necessary that the heteropolymeric protein be produced by one cell, or even in one culture. One alternative is to co-culture two cells, one of which produces one subunit and the other of which produces the other subunit; the secreted subunits can then associate in the medium to form heterodimer. Another alternative is to employ separate cultures, each producing a different subunit, and then to combine the culture media from them to permit association into heteropolymer.

Other host cells, vectors, promoters, transforming sequences, and viruses can also be employed. The host cell employed generally is dependent on the vector being used. For example, when the vector is replicating virus or non-replicating viral DNA, the host cells are cells capable of being infected or transfected, respectively, by those vectors; e.g., SV40-containing vectors require monkey host cells, preferably CV-1 cells. Where the cloning vector is a plasmid having procaryotic control sequences, prokaryotic host cells, e.g., E. coli, are used. Where the cloning vector is a plasmid having eukaryotic control sequences, appropriate eukaryotic host cells, e.g., mouse C127 cells, are used.

CLAIMS

1. A biologically active protein composed of a plurality of subunits, each said subunit being synthesized by a cell comprising an autonomously replicating expression vector comprising heterologous DNA encoding said subunit.

2. The protein of claim 1, at least one said subunit of said protein being synthesized by a eukaryotic cell, said protein being modified post-translationally.

3. The protein of claim 2, said post-translational modification being glycosylation.

4. The protein of claim 1, said protein being a secreted protein.

5. The protein of claim 3, said protein being a hormone.

6. The protein of claim 5, said hormone being a human fertility hormone.

7. A cell comprising a first autonomously replicating expression vector, said cell being capable of producing a biologically active heteropolymeric protein encoded at least in part by said first expression vector.

8. The cell of claim 7, said first expression vector encoding a first subunit of said heteropolymeric protein.

9. The cell of claim 7, further comprising a second autonomously replicating expression vector encoding a portion of said protein.

10. The cell of claim 9, a second subunit of said heteropolymeric protein being encoded by said second vector.

11. The cell of claim 7, two subunits of said heteropolymeric protein being encoded by said first expression vector.

12. The cell of claim 7 or claim 11, said heteropolymeric protein being a heterodimeric protein.

13. The cell of claim 7, said first vector being a plasmid.

14. The cell of claim 7, said cell being a mammalian cell.

15. The cell of claim 12, transcription of the two different subunits of said heterodimer being under the control of the SV40 late promoter.

16. The cell of claim 12, transcription of one subunit of said heterodimer being under the control of the SV40 early promoter, and transcription of the other subunit of said heterodimer being under the control of the mouse metallothionein promoter.

17. The cell of claim 16, said first vector comprising at least the 69% transforming region of the bovine papilloma virus genome.

18. The cell of claim 14, said cell being a monkey cell.

19. The cell of claim 14, said cell being a mouse cell.

20. An autonomously replicating expression vector encoding two different subunits of a heteropolymeric protein.

21. The expression vector of claim 20, said expression vector comprising a plasmid.

22. The expression vector of claim 20, said expression vector comprising a replicating virus.

23. The biologically active heteropolymeric protein produced by the cell of claim 7.

24. A method for producing a biologically active heteropolymeric protein comprising culturing host cells comprising a first autonomously replicating expression vector encoding at least a portion of said heteropolymeric protein.

25. The method of claim 24, a first subunit of said heteropolymeric protein being encoded by said first expression vector and a second subunit of said heteropolymeric protein being encoded by a second autonomously replicating expression vector comprised in said host cell.

26. The method of claim 24, at least two subunits of said heteropolymeric protein being encoded by said first expression vector.

27. The protein of claim 5, said hormone being thyroid stimulating hromone.

28. The cell of claim 12, said protein being thyroid stimulating hormone.

29. An autonomously replicating expression vector comprising two genes encoding two different heterologous proteins, said genes being under the control of two different promoters.

30. The expression vector of claim 29 wherein said two different promoters are derived from two different species.

31. The expression vector of claim 30 wherein one said promoter is of mammalian origin and the other said promoter is of viral origin.

32. The expression vector of claim 31 wherein said mammalian promoter is a metallothionein promoter and said viral promoter is the BPV promoter.

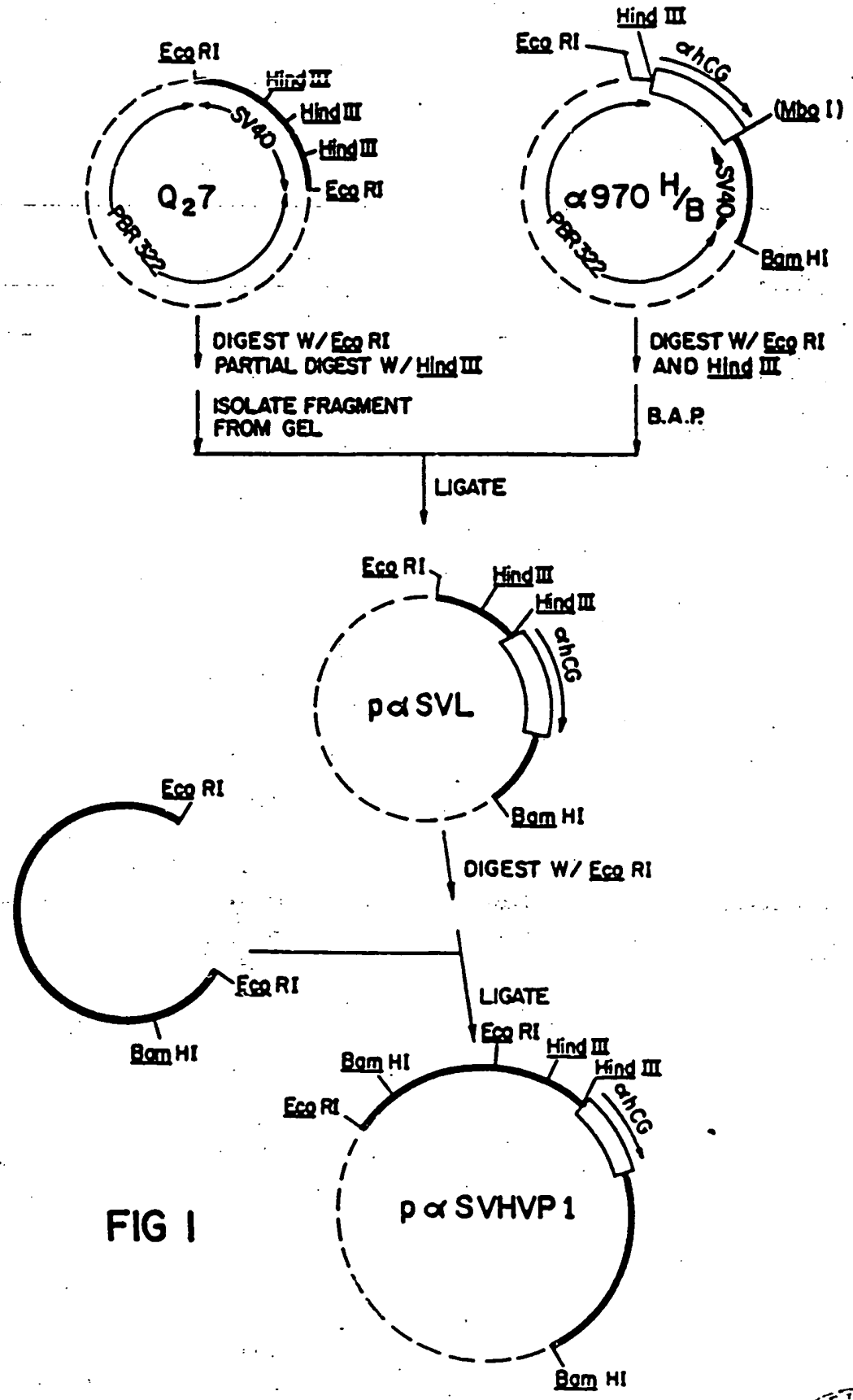


FIG 1

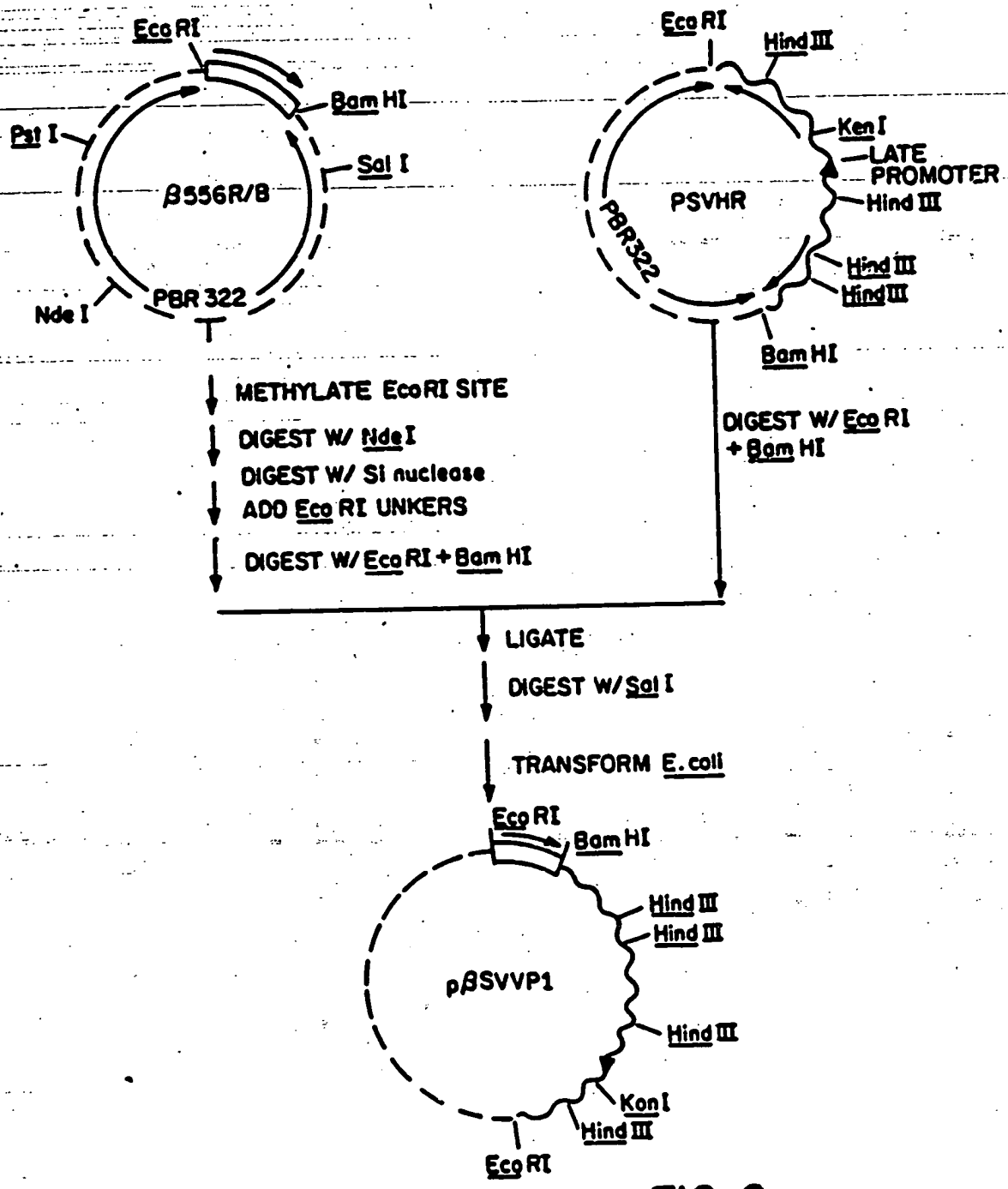


FIG. 2

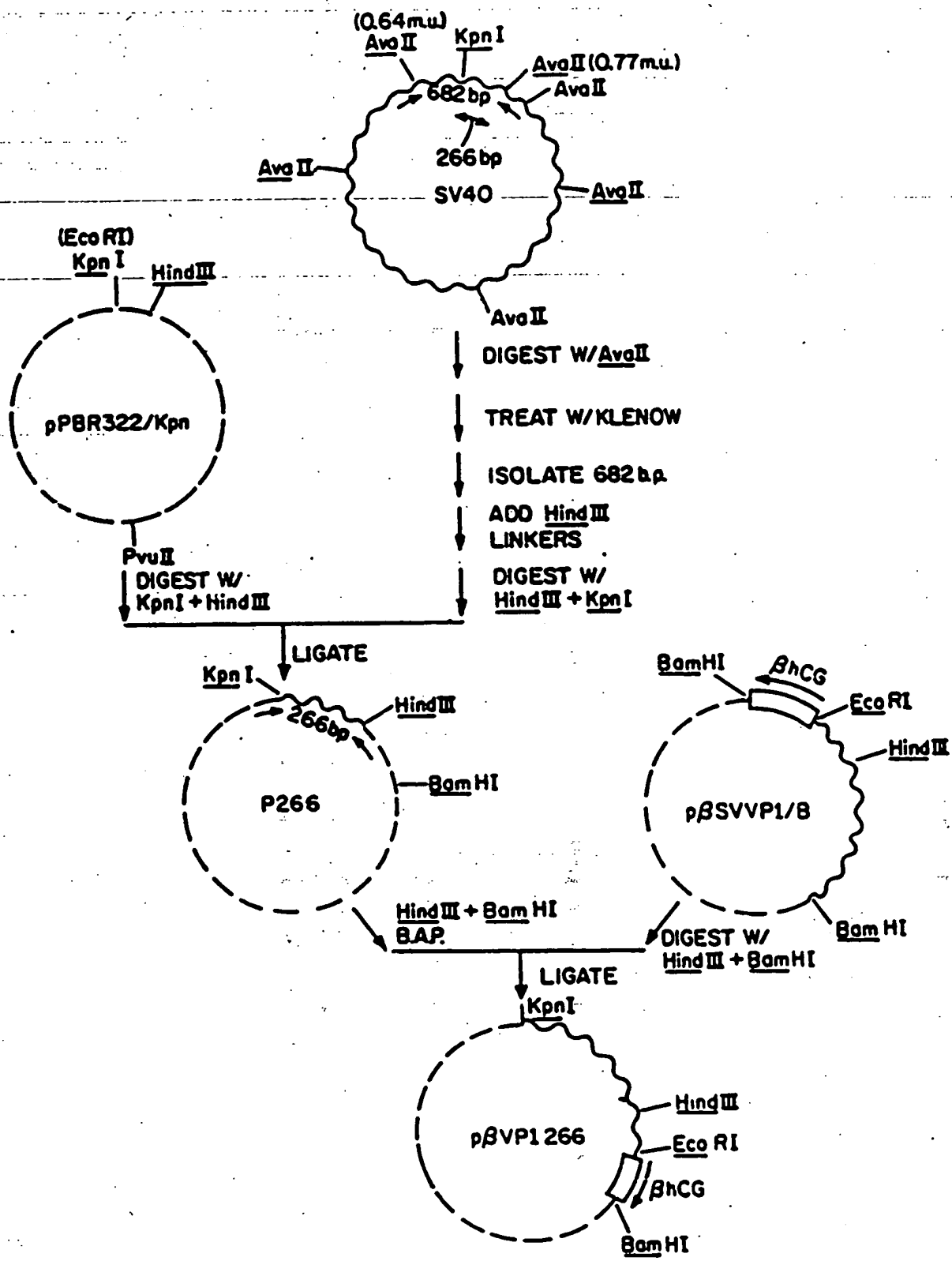


FIG. 3

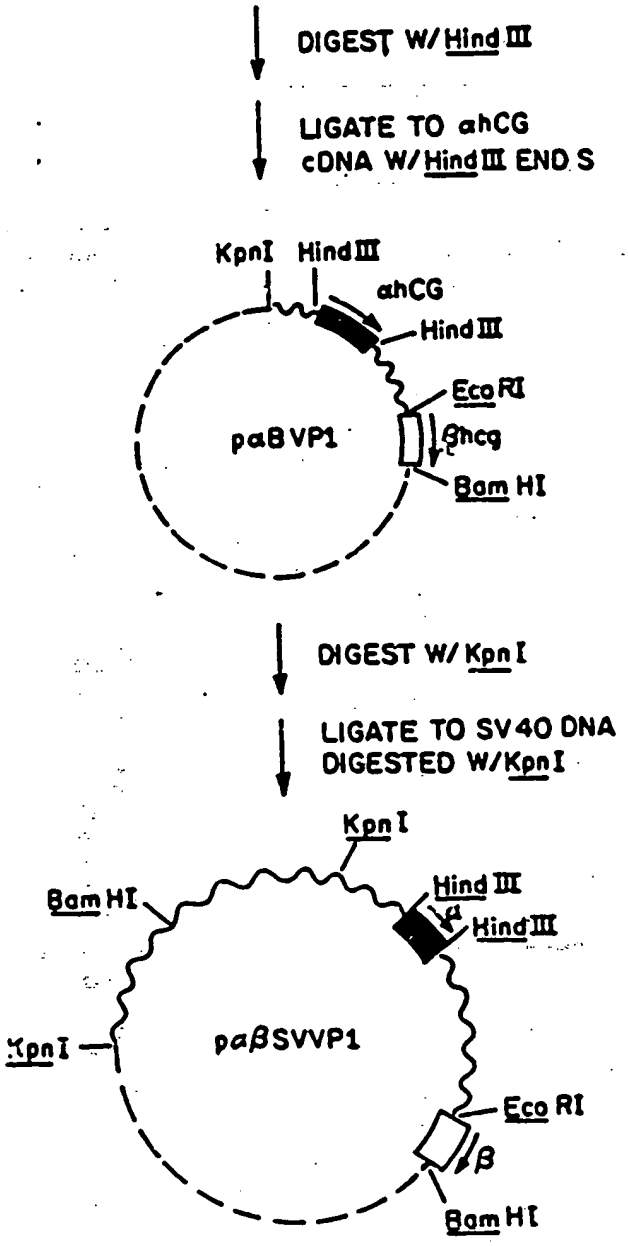


FIG. 3

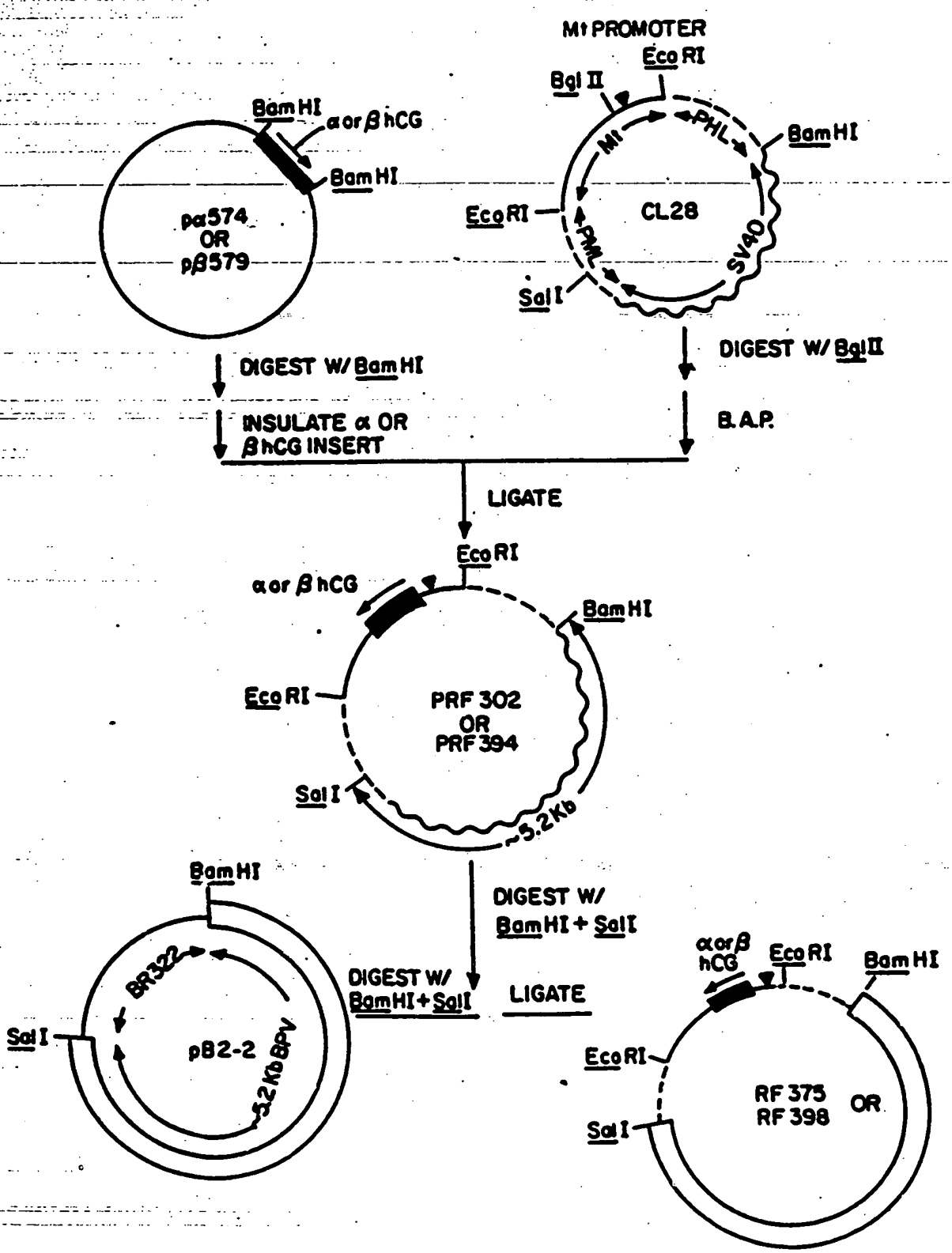

Mt PROMOTER
EcoRI
BglII
BamHI
αorβhCG
BamHI
pα574
OR
pβ579
MI
PHL
BamHI
EcoRI
CL28
PML
SV40
SalI
DIGEST W/ BamHI
DIGEST W/ BglII
INSULATE α OR
βhCG INSERT
B.A.P.
LIGATE
EcoRI
αorβhCG
BamHI
EcoRI
PRF 302
OR
PRF 394
SalI
5.2Kb
BamHI
DIGEST W/
BamHI + SalI
BR322
DIGEST W/
BamHI+SalI
LIGATE
αorβ
hCG
EcoRI
BamHI
SalI
pB2-2
5.2Kb BPV
EcoRI
RF 375
RF 398
OR
SalI

FIG. 4

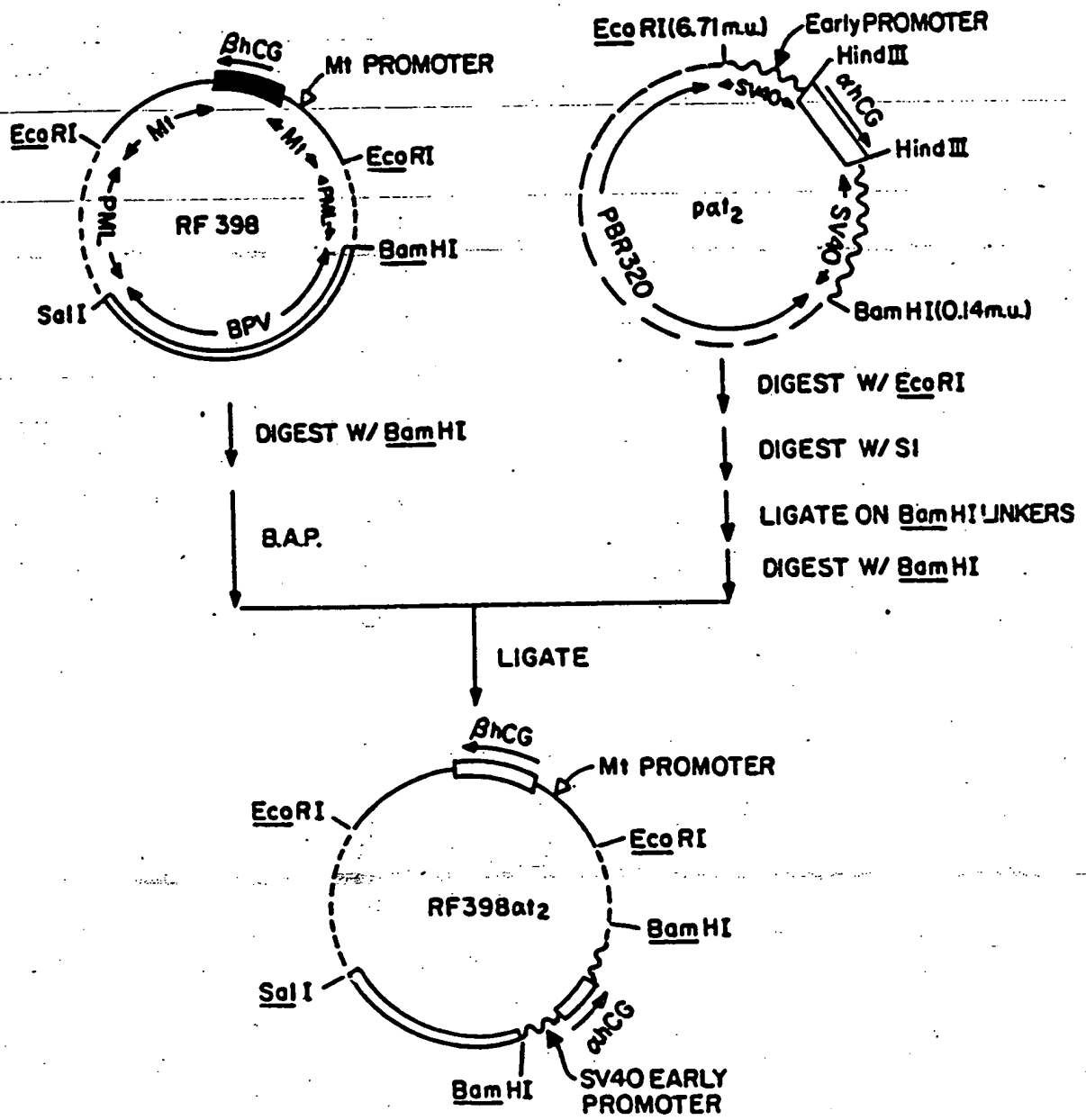


FIG. 5

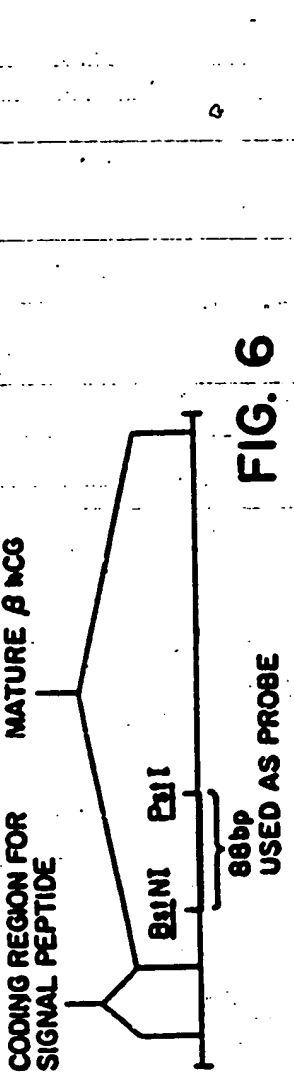


FIG. 6

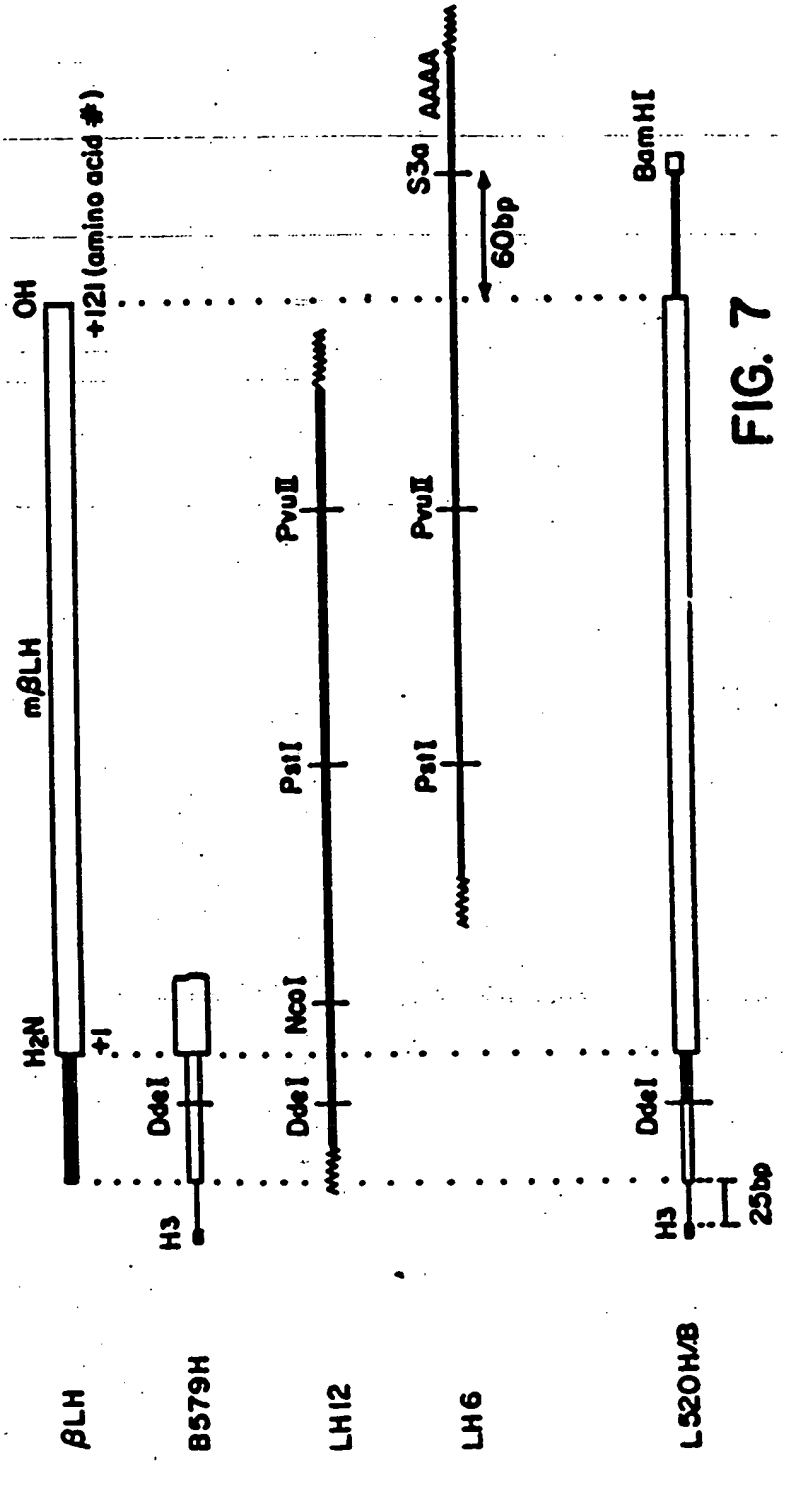


FIG. 7

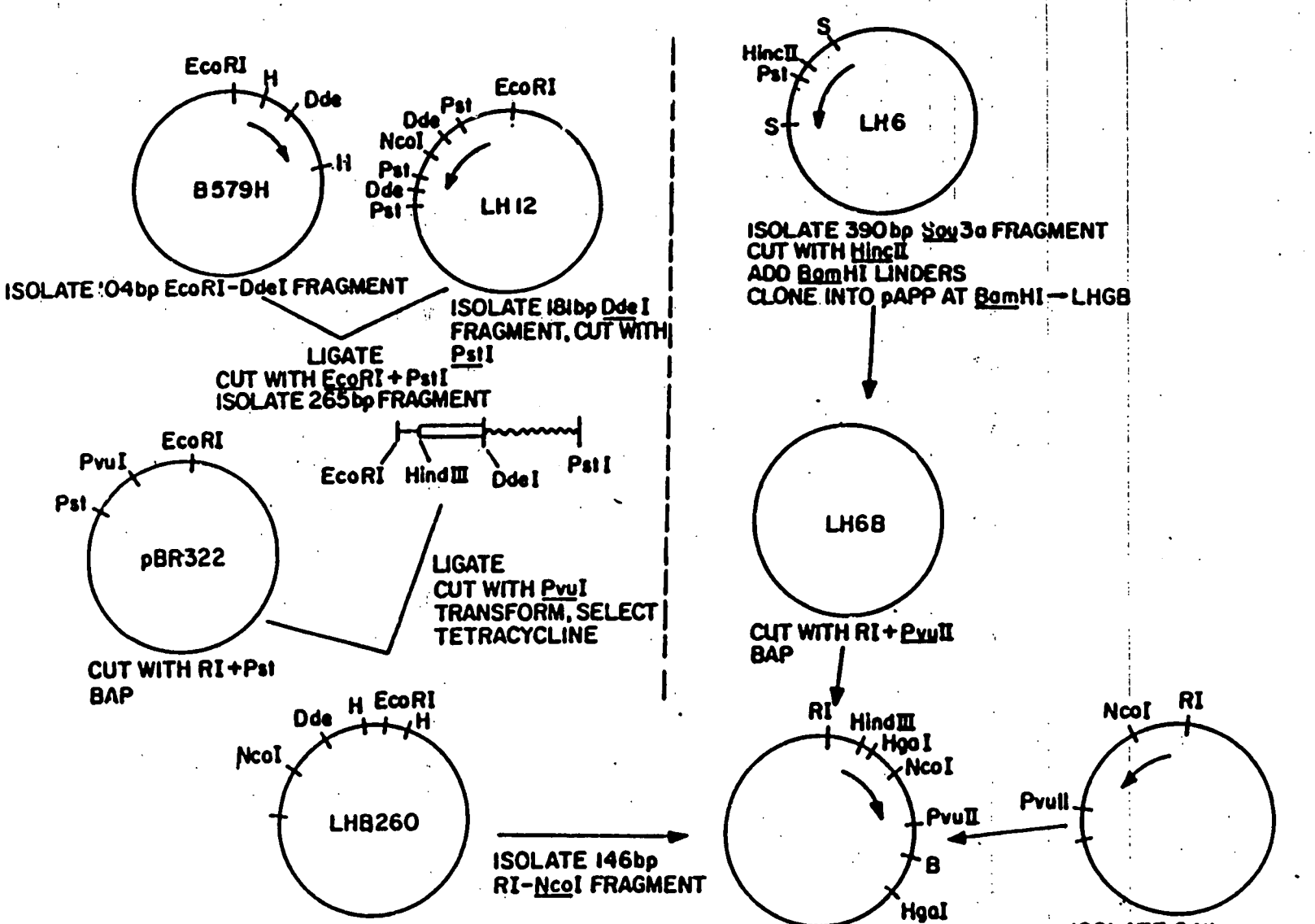
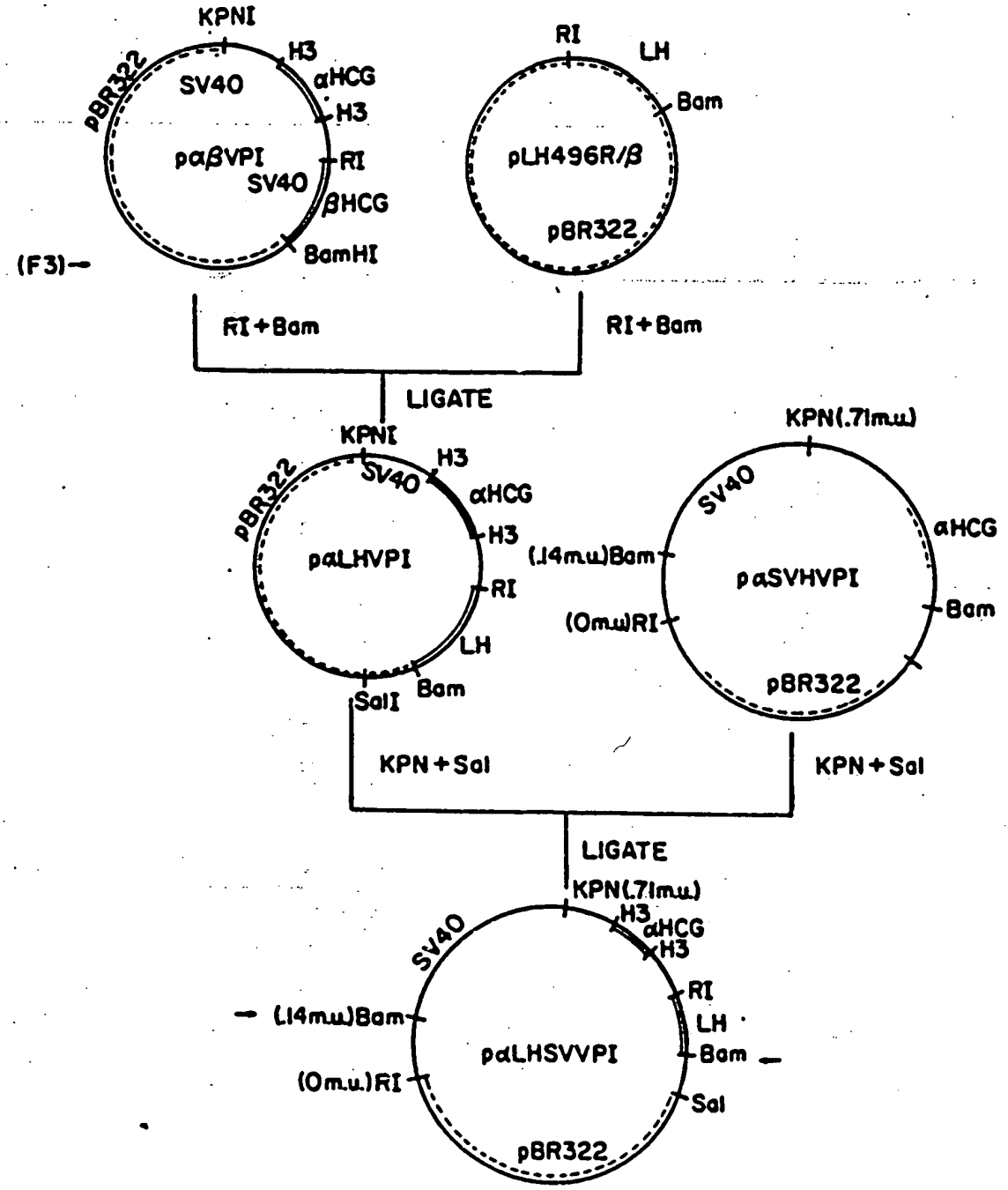


FIG. 8


KPNI
H3
αHCG
H3
SV40
pBR322
pαβVPI
RI
SV40
βHCG
BamHI
(F3)
RI
LH
Bam
pLH496R/β
pBR322
RI+Bam
RI+Bam
LIGATE
KPNI
H3
SV40
αHCG
H3
pBR322
pαLHVPI
RI
LH
SalI
Bam
KPN(.71m.u)
SV40
αHCG
(.14m.u)Bam
pαSVHVPI
Bam
(0m.u)RI
pBR322
KPN+Sal
KPN+Sal
LIGATE
KPN(.71m.u)
H3
αHCG
H3
SV40
RI
LH
(.14m.u)Bam
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Bam
(0m.u)RI
Sal
pBR322

FIG. 9

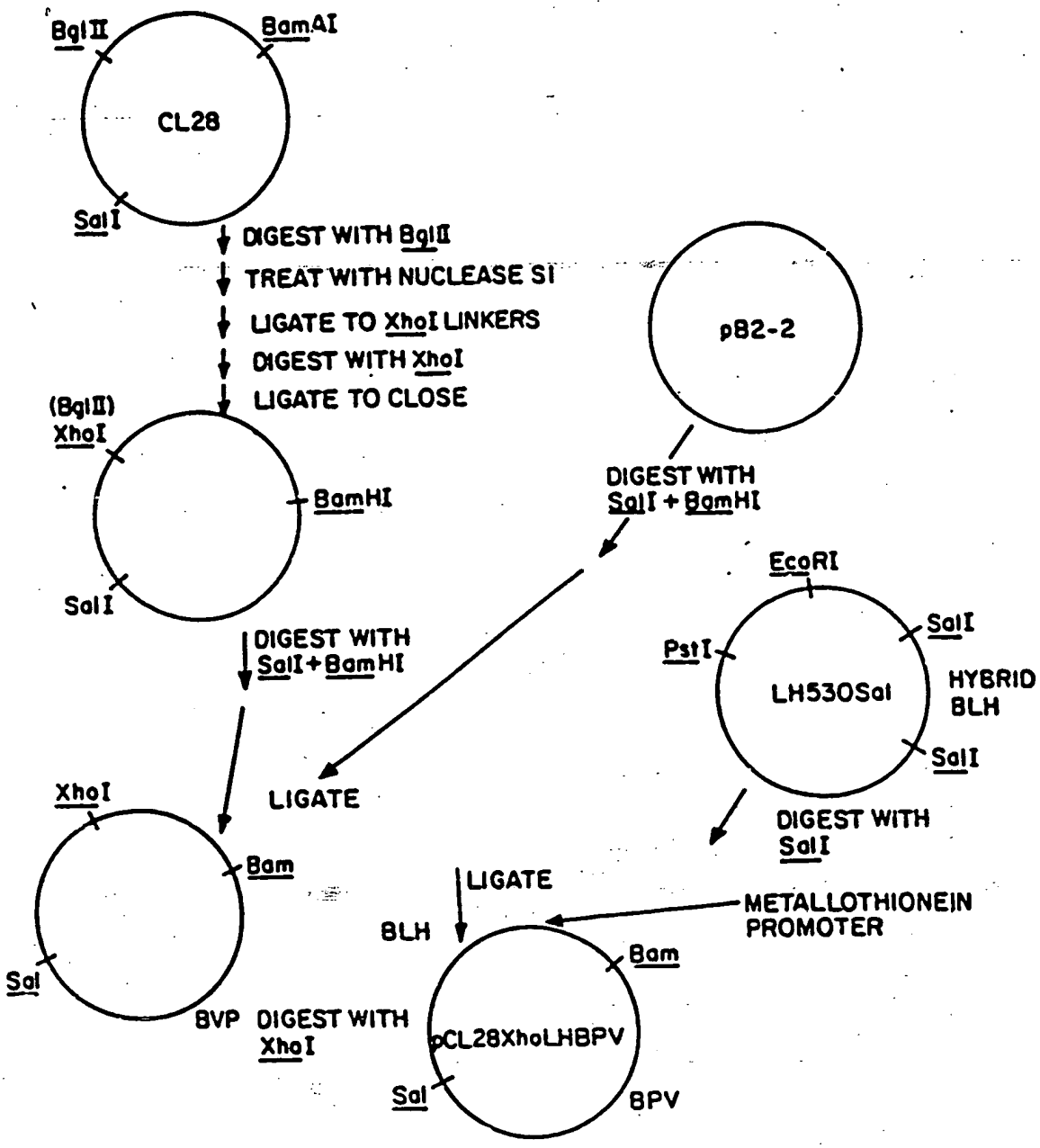
BglII
BamAI
CL28
SalI
DIGEST WITH BglII
TREAT WITH NUCLEASE S1
LIGATE TO XhoI LINKERS
DIGEST WITH XhoI
LIGATE TO CLOSE
(BglII)
XhoI
BamHI
SalI
pB2-2
DIGEST WITH
SalI + BamHI
EcoRI
SalI
PstI
LH530Sal
HYBRID
BLH
SalI
DIGEST WITH
SalI
DIGEST WITH
SalI+BamHI
XhoI
LIGATE
Bam
Sal
BVP
DIGEST WITH
XhoI
LIGATE
METALLOTHIONEIN
PROMOTER
BLH
Bam
pCL28XhoLHBPV
Sal
BPV

FIG. 10

INTERNATIONAL SEARCH REPORT

International Application No PCT/US84/01771

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL. -C12P 21/00,21/02, 21/04, C12N 15/00, 7/00, 5/00, 1/00 A61K 37/00,C07G 7/00

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/68, 70, 71, 172, 235, 240, 317 424/177 260/112R, 112.5

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

COMPUTER: CHEMICAL ABSTRACTS FILES 308, 309, 310, 311, 320
BIOSIS FILES: 5.55, 255
MEDLINE FILES: 152, 153, 154

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category [15]	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X	N, Proc. Natl. Acad.. Sci. USA Vol. 77, No. 6, pages 3149-3153 (June 1980)	1,3-6,27-28
Y	N, Proc. Natl. Acad. Sci USA Vol. 78, No. 9 pages 5329-5333 (September 1981)	1-32
Y	N, Molecular and Cellular Biology Vol. 1, No. 6 pages 486-496 (June 1981)	1-26, 29- 32
Y	N, Proc. Natl. Acad. Sci. USA Vol. 79, pages 4897-4901 (August 1982)	1-26,29-32
Y	N, Proc. Natl. Acad Sci. USA Vol. 79 pages 4030-4034 (August 1982)	1- 26,29-32
Y	N, Nature Vol. 286 pages 684-687 (August 1980)	1-26,29-32
Y	N, Nature Vol. 281 pages 351-356 (October 1979)	1-26, 29-32
Y	N, Proc. Natl. Acad. Sci. USA, Vol. 78, No. 4, pages 2606-2610 (April 1981)	1-26,29-32

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
28 January 1985	12 FEB 1985
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	Dinah H. Lewitan Dinah H. Lewitan

Form PCT/ISA/210 (second sheet) (October 1981)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	N, Proc. Natl. Acad. Sci. USA Vol. 50 pages 397-401 (January 1983)	1-26,29-32
T	N, J. Molecular and Applied Genetics Vol. 1 pages 3-18 (1981)	1-32
A	N, Science Vol. 200 pages 494-502 (May 1978)	15, 16
Y	N, Virology Vol. 117 pages 536-540 (1982)	1-26, 29-32
P	US, A, 4,419,446 Published 6 December 1983	1-26, 29-32

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.